

1 **Decreasing pdzd8-mediated mitochondrial-ER contacts in neurons**
2 **improves fitness by increasing mitophagy**

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18

19 **Abstract**

20 The complex cellular architecture of neurons combined with their longevity makes
21 maintaining a healthy mitochondrial network particularly important and challenging.
22 One of the many roles of mitochondrial-ER contact sites (MERCs) is to mediate
23 mitochondrial quality control through regulating mitochondrial turn over. Pdzd8 is a
24 newly discovered MERC protein, the organismal functions of which have not yet been
25 explored. Here we identify and provide the first functional characterization of the
26 *Drosophila melanogaster* ortholog of Pdzd8. We find that reducing pdzd8-mediated
27 MERCs in neurons slows age-associated decline in locomotor activity and increases
28 lifespan in *Drosophila*. The protective effects of pdzd8 knockdown in neurons correlate
29 with an increase in mitophagy, suggesting that increased mitochondrial turnover may
30 support healthy aging of neurons. In contrast, increasing MERCs by expressing a
31 constitutive, synthetic ER-mitochondria tether disrupts mitochondrial transport and
32 synapse formation, accelerates age-related decline in locomotion and reduces lifespan.
33 We also show that depletion of pdzd8 rescues the locomotor defects characterizing an
34 Alzheimer's disease (AD) fly model over-expressing Amyloid β_{1-42} (A β_{42}) and
35 prolongs the survival of flies fed with mitochondrial toxins. Together, our results
36 provide the first *in vivo* evidence that MERCs mediated by the tethering protein pdzd8
37 play a critical role in the regulation of mitochondrial quality control and neuronal
38 homeostasis.

39 **Keywords**

40 Mitochondria, ER (Endoplasmic reticulum), organelle contact site, mitoQC, Amyloid
41 beta, mitophagy, lifespan, *Drosophila melanogaster*, aging, neurodegeneration,
42 healthspan, MERCs, Alzheimer's disease.

43 **Introduction**

44 Since the vast majority of neurons are postmitotic, maintaining functional neurons
45 throughout an organism's lifetime requires tight regulation of organelle functions and
46 stress responses. Mitochondria and the endoplasmic reticulum (ER) extend throughout
47 neuronal processes including axons and dendrites, and both are vital and interdependent
48 contributors to neuronal health (Wu *et al*, 2017). Mitochondria-ER contacts (MERCs)
49 are controlled by a variety of contact site proteins and contribute to a range of functions
50 required for proper development and maintenance of postmitotic neurons, including
51 regulation of calcium homeostasis, lipid biogenesis, organelle reshaping and dynamics,
52 and metabolic signalling (Giacomello & Pellegrini, 2016; Paillusson *et al*, 2016).
53 Dysregulation of MERCs is particularly damaging to neurons as they are particularly
54 susceptible to calcium overload, oxidative and ER stresses and to altered mitochondrial
55 function, localization and transport (Lee *et al*, 2018a; Misgeld & Schwarz, 2017).

56
57 As MERCs are modulated by a number of different protein complexes, the detrimental
58 effects of dysregulation of MERCs are varied due to the diversity of these contact site
59 functions (Martino Adami *et al*, 2019). The critical function of MERCs in regulating
60 cellular responses to damage and stress is underscored by the finding that many human
61 patient cellular models and animal models of age-related neurodegenerative diseases
62 have been shown to have disrupted MERCs. Both reduced MERCs (De Vos &
63 Hafezparast; Sepulveda-Falla *et al*, 2014) and increased MERCs (Area-Gomez *et al*,
64 2012; Gómez-Suaga *et al*, 2019; Zampese *et al*, 2011) have been implicated in
65 neurodegenerative diseases. Consequently, there is still little consensus on how altered
66 MERCs contribute to neurodegeneration, even within a single disease model

67 (Erpapazoglou *et al*, 2017). The various functions of MERCs make it likely that
68 multiple mechanisms might be involved and, with an ever-expanding toolkit, we can
69 now better define the molecular identities and specific functions of ER-mitochondria
70 tethering complexes and begin to unify many of the seemingly conflicting discoveries
71 in this rapidly growing field (Csordas *et al*, 2018).

72
73 Pdzd8 is one of the most recently discovered proteins that mediates mammalian
74 MERCs (Hirabayashi *et al*, 2017) and is a paralog of Mmm1 (Wideman *et al*, 2018), a
75 component of the fungal-specific ER mitochondria encounter structure (ERMES) and
76 first MERC complex identified (Kornmann *et al*, 2009). We used *Drosophila*
77 *melanogaster* to study the consequences of neuronal depletion of pdzd8 both at the
78 cellular and at the organismal level. Importantly, we also describe how the phenotypes
79 associated with neuron-specific depletion of pdzd8 change with age and may contribute
80 to healthy aging. We show the MERCs mediated by the pdzd8 tethering protein regulate
81 mitochondrial turnover through mitophagy and that reducing these contacts prolonged
82 locomotor activity and lifespan in *Drosophila melanogaster*.

83

84 **Results**

85

86 **Characterization of fly pdzd8**

87 The *Drosophila melanogaster* gene *CG10362* encodes an uncharacterized protein in
88 the PDZK8 family (Lee & Hong, 2006). *CG10362* has a similar predicted domain
89 structure to mammalian Pdzd8 (Figure 1A). Expression of *CG10362* in flies is low but
90 is most highly expressed in the central nervous system (FlyAtlas 2; Figure S1A)(Leader
91 *et al*, 2018) and is enriched in neurons over glia (Figure S1B) (Davie *et al*, 2018). This
92 specificity in expression in *Drosophila* provided an excellent opportunity to explore the
93 neuronal functions of this newly discovered MERC protein and also to investigate the
94 functional relevance of MERCs in adult and aging neurons. We propose that *CG10362*
95 encodes the fly ortholog of mammalian Pdzd8 and, based on the data presented in this
96 paper, we propose to name it *pdzd8*.

97

98 To characterize the function of pdzd8 in flies, we used the UAS-GAL4 system to
99 manipulate its expression (Brand & Perrimon, 1993). Ubiquitous expression of an
100 RNAi construct targeting *pdzd8* strongly reduces its mRNA levels in larvae (Figure
101 S1C). To establish that MERCs were decreased in neurons expressing *pdzd8*-RNAi, we
102 analysed adult fly brains by transmission electron microscopy (TEM) and manually
103 identified contacts between ER and mitochondria (Figure 1B). Accordingly, the
104 proportion of mitochondria in the soma of adult fly neurons in contact with ER is
105 reduced in flies expressing *pdzd8*-RNAi (Figure 1C and S1F).

106

107 To confirm that *pdzd8*-RNAi reduces MERCs in axons as well as soma, we adapted the
108 recently developed MERC quantification tool, a split-GFP-based contact site sensor
109 (SPLICS) (Cieri *et al*, 2017) to create a SPLICS transgenic reporter line. The SPLICS
110 construct targets β -strands 1-10 of GFP to the mitochondrial outer membrane and β -
111 strand 11 to the ER membrane, and where these membranes are in close proximity
112 fluorescent puncta are produced by reconstitution of the split-GFP (Figure S2A). To
113 validate this tool in *Drosophila*, we expressed SPLICS in motor neurons (Figure S2B)
114 and compared the number of puncta in control axons and those expressing a well
115 characterized artificial ER-mitochondrial tether developed by Csordas *et al*. (Basso *et*

116 *al*, 2018; Csordas *et al*, 2006). The tether construct induces formation of ~5 nm MERCs
117 through transmembrane domains that anchor it in both the mitochondrial outer
118 membrane and the ER (Figure S2C). The density of SPLICS puncta in the axons
119 expressing the tether was four times higher than controls (Figure S2D), indicating the
120 SPLICS reporter was able to detect the increased MERCs resulting from synthetic
121 tether expression in neurons *in vivo*.

122
123 Using this SPLICS construct we also detected a significant decrease in the density of
124 SPLICS puncta in central larval axons (bundles projecting to segments A7 and A8)
125 expressing the *pdzd8*-RNAi compared to *LacZ*-RNAi controls (Figure 1D-E). While
126 *pdzd8* expression is low beyond the nervous system, ubiquitous knockdown of *pdzd8*
127 also reduces the extent of MERCs measured by fluorescence colocalization of ER and
128 mitochondrial signals by super-resolution microscopy (structured illumination
129 microscopy, SIM) in larval epidermal cells (Figure 1F-G and S1E), corroborating our
130 results using TEM and SPLICS analysis.

131
132 Consistent with the first reported function of Pdzd8 at MERCs in mouse neurons
133 (Hirabayashi *et al*, 2017), we observed a reduced number of MERCs (Figure 1), but no
134 obvious changes in mitochondrial or ER morphology in fly larval or adult neurons upon
135 *pdzd8* knockdown (See Figures 1B, 1D, 1F, S1D-E, 4A-B, 4F-I, 5A and 5C). Together,
136 these data show that *CG10362* encodes the *Drosophila* homolog of Pdzd8 and functions
137 like mammalian Pdzd8 to regulate MERCs. We next sought to investigate the
138 consequences of loss of *pdzd8* from neurons on organismal phenotypes.

139

140 **Reduced mitochondria-ER contacts are protective in aging neurons**

141 Knockdown of *pdzd8* in fly neurons produces viable adults. There was no impact on
142 the locomotor performance of pan-neuronal *pdzd8*-RNAi knockdown in young flies
143 assessed in a climbing assay (Figure 2A). Surprisingly, we found that loss of *pdzd8*
144 dramatically slowed the age-associated decline in locomotor activity assessed by the
145 climbing assay (Figure 2A). Importantly, this effect was reproduced by motor neuron-
146 specific *pdzd8* knockdown (Figure S3A). Strikingly, the increase in locomotor activity
147 was accompanied by a significant increase in lifespan (Figure 2C).

148

149 In contrast, increasing MERCs by expression of a synthetic mitochondria-ER tether in
150 all neurons resulted in a climbing defect in young flies and a significant acceleration of
151 the age-related decline in climbing (Figure 2B), consistent with previous reports (Basso
152 *et al*, 2018). This climbing defect was exacerbated with age (Figure 2B) and associated
153 with a substantially reduced lifespan (Figure 2D). Consistent with these results,
154 increased expression of *pdzd8* also resulted in decreased climbing ability with age
155 (Figure S3B). Notably, this effect was suppressed by co-expression of the *pdzd8*-RNAi,
156 further validating the specificity of this transgene (Figure S3B). Therefore, decreasing
157 *pdzd8*-mediated MERCs in neurons prolonged lifespan and protected against
158 locomotor decline with age, while increased MERCs in neurons were found to speed
159 up the age-related decrease in locomotor activity and decreased lifespan.

160

161 **Loss of neuronal *pdzd8* promotes survival in the presence of mitochondrial toxins**

162 To investigate how reduction of *pdzd8*-mediated MERCs improved fitness i.e.
163 prevented age-related decline in locomotor activity and increased lifespan, we assessed
164 whether reducing *pdzd8* expression in neurons may protect from additional stresses
165 during aging. We assessed lifespan in flies aged on food with limited nutrients (5 %

166 sucrose, 1 % agar), and found that in contrast to flies aged on a rich diet (Figure 2C),
167 neuronal expression of *pdzd8*-RNAi no longer extended the lifespan in comparison to
168 controls (Figure 3A). When an additional oxidative stress was introduced by adding
169 hydrogen peroxide to the food, the flies expressing *pdzd8*-RNAi died faster than
170 controls (Figure 3B). Therefore, in the presence of general stresses, *pdzd8*-RNAi is also
171 not protective.

172
173 Due to the function of *pdzd8* at MERCs, we examined whether the protective effects
174 caused by the *pdzd8* depletion in neurons were associated with mitochondrial damage.
175 To address this, we fed the flies mitochondrial toxins: Rotenone, a complex I inhibitor
176 or antimycin A, a complex III inhibitor, both block the electron transport chain and
177 result in dysfunctional mitochondria. Reducing *pdzd8* levels in neurons significantly
178 prolonged the survival of flies fed with mitochondrial toxins rotenone (Figure 3C) or
179 antimycin A (Figure 3D) compared to control flies. As improved mitochondrial
180 function could also contribute to the protective effects of loss of *pdzd8* in neurons we
181 measured ATP levels in young or aged fly heads expressing *pdzd8*-RNAi but found no
182 significant differences (Figure S3D). These results indicated that neuronal loss of *pdzd8*
183 protects flies from damage induced by mitochondrial toxins.

184

185 **Modulating MERCs causes axonal transport and NMJ defects**

186 While mitochondrial motility is important for neuronal health, it remains an open
187 question whether decline of mitochondrial transport in neurons contributes to aging
188 (reviewed in (Mattedi and Vagnoni 2019)). We first tested the hypothesis that decreased
189 ER-mitochondrial tethering contributes to the protective effect of *pdzd8*
190 downregulation in aging through changes in mitochondrial motility. We examined the
191 distribution and morphology of mitochondria in axons of CCAP efferent neurons (Park
192 *et al.*, 2003). We found no significant change in mitochondrial length or density in larval
193 axons when comparing control to *pdzd8*-RNAi or synthetic ER-mitochondria tether-
194 expressing neurons (Figure 4A-C). However, increasing tethering dramatically
195 decreased mitochondrial motility (Figure 4D-E). In contrast, knockdown of *pdzd8* to
196 reduce MERCs had no effect on axonal transport in larvae (Figure 4D-E).

197

198 To better understand the effects of altered MERCs in neurons, we analysed the
199 morphology of mitochondria located in 1s boutons of larval neuromuscular junctions
200 (NMJs) on muscle 4. Knockdown of *pdzd8* led to smaller NMJs and a significant
201 reduction of mitochondrial volume, but overall no change in mitochondrial density
202 compared to control flies (Figure 4F-G), showing that mitochondria distribute normally
203 in these smaller NMJs. Increased tethering, however, resulted in severely deformed
204 NMJs (Figure S3C) and made NMJs type 1s and 1b synaptic boutons indistinguishable,
205 making it impossible to quantify mitochondrial density in 1s boutons. Together, these
206 results show that increasing tethering has dramatic and detrimental effects early in
207 development but reduced tethering through *pdzd8*-RNAi expression has more limited
208 effects during these early stages of neuronal development.

209

210 **Reducing *pdzd8* expression increases mitophagy in aged neurons**

211 We hypothesized that the reduced sensitivity to mitochondrial toxins may be due to
212 improved mitochondrial quality control mechanisms. Thus, we analysed the levels of
213 mitophagy, the clearance of damaged mitochondria by autophagy, in these neurons
214 using the mitoQC mitophagy reporter (Allen *et al.*, 2013; Lee *et al.*, 2018b). This reporter
215 is a pH-sensitive mCherry-GFP fusion targeted to the mitochondrial outer membrane

216 which provides a read out of mitophagy in the form of mCherry-only signal where the
217 acidic lysosomal environment has quenched the GFP. Mitophagy was detected in the
218 soma of nSyb-GAL4-expressing neurons in both larval and adult brains (Figure 5A and
219 C). There was no significant difference in mitophagy levels in larval neurons expressing
220 *pdzd8*-RNAi or control (Figure 5A-B). Quantification of mitoQC mCherry puncta did
221 not indicate an age-dependent increase in mitophagy in the brains of control adults,
222 however mitophagy was significantly increased in the brains of 20-day-old *pdzd8*-
223 RNAi animals compared to controls (Fig 5C-D).

224

225 The majority of mitochondria in neurons are found in the neurites, so to examine
226 mitophagy in axons of aged flies, we analysed mitoQC signal in axons of the adult fly
227 wing *in situ* (Vagnoni & Bullock, 2016) (Figure 5E). Here, in contrast to the adult brain
228 cell bodies, we did observe an age dependent increase in mitophagy in axons of control
229 flies (Figure 5F). Consistent with our previous results, *pdzd8* knockdown further
230 increased mitophagy in axons of aged flies (Figure 5E-F). Together, these results show
231 that loss of *pdzd8* promotes the turnover of mitochondria in aging neurons.

232

233 **Reduced MERCs is protective in a fly model of Alzheimer's disease**

234 Our results indicate that the loss of *pdzd8* is protective against mitochondrial insults,
235 prevents the age-related decrease in locomotion and increases lifespan. Since
236 mitochondrial dysfunction is a common feature of many neurodegenerative diseases,
237 and altered MERCs has been documented in some, we next sought to explore the
238 neuroprotective potential of *pdzd8* depletion in an age-related neurodegenerative
239 disease model. To test this, we turned to an Alzheimer's disease fly model where the
240 expression of pathogenic A β ₄₂ has been shown to cause neural dysfunction, due in part
241 to oxidative stress (Rival *et al*, 2009).

242

243 Since increased MERCs have also been associated with Alzheimer's disease (Area-
244 Gomez *et al*, 2012; Del Prete *et al*, 2017), we first used the SPLICS reporter to
245 determine the number of MERCs in larval axons. Consistent with other cellular and
246 organismal models, flies expressing the A β ₄₂ showed an increase in SPLICS puncta,
247 indicating MERCs are increased in axons of this model of Alzheimer's disease (Figure
248 6A-B). Flies expressing A β ₄₂ in neurons exhibit a significant climbing defect in young
249 flies that worsens rapidly with age (Figure 6C)(Crowther *et al*, 2005). However, pan-
250 neuronal *pdzd8* knockdown in combination with A β ₄₂ was sufficient to substantially
251 ameliorate the decline in locomotor activity observed in young and 10-day-old A β ₄₂
252 flies (Figure 6C). Thus, reducing *pdzd8*-mediated MERCs is protective in this
253 progressive neurodegenerative disease model with increased MERCs.

254 **Discussion**

255

256 Here we have identified and characterized the putative *Drosophila* homolog of the
257 newly discovered mammalian MERC tethering protein Pdzd8 (Hirabayashi *et al*,
258 2017). The sequence divergence between Pdzd8 and its yeast paralog Mmm1 and the
259 additional domains present in Pdzd8 (Wong & Levine, 2017), made the relationship
260 between these paralogs difficult to identify (Wideman *et al*, 2018). While the conserved
261 predicted domain structure strongly suggests *Drosophila* CG10362 encodes the fly
262 homolog of mouse Pdzd8, the low, 21 % overall sequence identity of these proteins
263 raises the interesting possibility of evolution of more species-specific functional
264 specialisation.

265

266 Using RNAi, we characterized the effects of depletion of *pdzd8* in *Drosophila* with a
267 focus on neurons where this protein is most highly expressed. Knockdown of *pdzd8*
268 reduces contacts between the ER and mitochondria in epidermal cells measured using
269 super resolution microscopy of ER and mitochondria labeled with fluorescent reporters,
270 in motor neurons, monitored by the contact site reporter SPLICS, and in the soma of
271 adult neurons using transmission electron microscopy. These data suggest that *pdzd8*,
272 like its mammalian ortholog, functions as a tether between ER and mitochondria. The
273 only other neuronally expressed tethering protein that has been characterized in flies is
274 the *Drosophila* ortholog of Mfn2, Marf (Hwa *et al*, 2002). However, the analysis of
275 Marf is complicated by its additional roles in mitochondrial and ER morphology
276 (Debattisti *et al*, 2014; El Fissi *et al*, 2018; Sandoval *et al*, 2014). While mammalian
277 Pdzd8 is expressed in a range of tissues (Hirabayashi *et al*, 2017), in flies, *pdzd8* mRNA
278 expression is low outside the nervous system. Knockdown of *pdzd8* and expression of
279 a synthetic tether therefore provided a unique opportunity to simply and selectively
280 examine the function of MERCs in *Drosophila* neurons.

281

282 We found increased ER-mitochondrial tethering in neurons strongly impairs climbing
283 ability and reduces lifespan of flies. Such detrimental effects are consistent with
284 previous reports using this line (Basso *et al*, 2018). Other similar manipulations have
285 also been shown to result in dopaminergic neuron loss (Lee *et al*, 2018c) and
286 detrimental effects on sleep in ventral lateral neurons (Valadas *et al*, 2018). Here we
287 also show highly abnormal NMJ development, with increased MERCs and smaller, but
288 otherwise structurally intact NMJs upon *pdzd8* knockdown. The function of the yeast
289 paralog Mmm1 and of its Synaptotagmin-like mitochondrial lipid-binding protein
290 (SMP) domains, suggests that disruptions in lipid transfer due to less *pdzd8* might
291 contribute to these developmental defects (Jeong *et al*, 2017; Kawano *et al*, 2018;
292 Shirane *et al*, 2020), however whether lipid biogenesis defects could contribute to a
293 protective effect of *pdzd8*-RNAi in neurons remains an open question.

294

295 Transport of mitochondria is intimately linked to the health of neurons, at least in the
296 peripheral nervous system (De Vos *et al*, 2008; Harbauer, 2017; Misgeld & Schwarz,
297 2017). Although there is some evidence that increased MERCs may be directly
298 associated with decreased mitochondrial motility (Krols *et al*, 2018), this has not been
299 shown in neurons which are particularly sensitive to mitochondrial transport imbalance
300 (Maday *et al*, 2014). Our data suggests axonal mitochondrial transport defect
301 contributes to the detrimental effects of increased tethering and adds to the evidence
302 that efficient mitochondrial transport is essential for healthy aging neurons, as seen in
303 many models of neurodegenerative motor disorders (Baldwin *et al*, 2016).

304

305 In contrast to the detrimental effects of increased tethering, reducing MERCs by
306 knockdown of *pdzd8* in *Drosophila* neurons dramatically delayed age-associated
307 decline in locomotor activity and significantly extended median lifespan compared to
308 control animals. Since decline in mitochondrial transport is proposed to contribute to
309 neuronal aging (Vagnoni & Bullock, 2018), we hypothesized that reducing tethering in
310 the aging flies might be protective by allowing sustained mitochondrial motility
311 (Mattedi & Vagnoni, 2019). However, we detected no change in the percentage of
312 motile mitochondria in larval neurons with reduced *pdzd8* expression. Since
313 knockdown of *pdzd8* also prolonged the survival of flies fed mitochondrial toxins, this
314 suggests that the protective effects of reducing MERCs might instead be result from

315 more efficient clearance of the damaged mitochondria accumulating with age or more
316 acutely by feeding flies mitochondrial toxins.

317

318 To explore the clearance of damaged mitochondria, we examined the age dependence
319 of mitophagy using the mitoQC reporter. Clearance of damaged cellular components
320 by autophagy is critical for the maintenance of healthy neurons with age (Stavoe &
321 Holzbaur, 2019). More specifically, the clearance of damaged mitochondria via
322 mitophagy is also thought to be a key factor in healthy aging of neurons (Ma *et al*, 2018;
323 Pickrell & Youle, 2015; Whitworth & Pallanck, 2017). It remains unclear, however
324 whether, increased or decreased mitophagy in neurons is protective during aging
325 (Montava-Garriga & Ganley, 2020) and if specific neuronal populations are more
326 susceptible to mitochondrial turnover. Studies examining mitophagy in aging brains
327 have produced inconsistent results, with differences observed between brain regions
328 and cell types (Cornelissen *et al*, 2018; Lee *et al*, 2018b; Sun *et al*, 2015). Consistent
329 with our previous result in dopaminergic neurons (Lee *et al*, 2018b), we also did not
330 detect any age-related change in mitophagy levels in the cell bodies in the brains of
331 control flies.

332

333 There is accumulating evidence showing that axonal mitophagy occurs in cultured
334 mammalian neurons (Ashrafi *et al*, 2014; Zheng *et al*, 2019), but other results suggest
335 axonal maintenance in flies may not require mitophagy (Cao *et al*, 2017). There is
336 evidence of mitophagy *in vivo* in neurites in young mice (McWilliams *et al*, 2018;
337 McWilliams *et al*, 2019), but mitoQC has not previously been used to detect mitophagy
338 in neurites during aging (Cornelissen *et al*, 2018). Using mitoQC, we detected a
339 significant amount of mitophagy in wing axons of control flies which increased with
340 age. Our results suggest mitophagy in axon but not cell bodies increases with age, but
341 future experiments should specifically examine variations in mitophagy with age across
342 brain regions, cell types and even other subcellular compartments such as dendrites.

343

344 Here we provide the first *in vivo* evidence that mitophagy may be regulated by pdzd8-
345 mediated MERCs. Mitophagy levels did not change in young flies with less pdzd8, but
346 when aged, these flies displayed significant increases in mitophagy in both soma and
347 axons. There is also accumulating evidence that defects in mitophagy contribute to the
348 early stages of Alzheimer's disease (AD) (Kerr *et al*, 2017; Lee *et al*, 2019) and
349 boosting mitophagy has been shown to be protective in worm and mouse AD models
350 (Fang *et al*, 2019). Here we show increased MERCs in an AD fly model consistent with
351 the altered MERCs identified in both patients and cell models of AD (Area-Gomez *et al*,
352 2012). Expression of *pdzd8*-RNAi in the AD flies significantly slowed their age-
353 associated decline in climbing, consistent with reduced MERCs allowing more efficient
354 mitophagy and removal of damaged mitochondria.

355

356 Mitochondrial dysfunction and associated metabolic changes are considered a hallmark
357 of aging (Lopez-Otin *et al*, 2013). Boosting mitophagy has been suggested to increase
358 lifespan and protect animals from mitochondrial toxins in both *D. melanogaster*
359 (Aparicio *et al*, 2019; Rana *et al*, 2017) and *C. elegans* (Palikaras *et al*, 2015). Early
360 work on starvation induced autophagosome biogenesis showed MERCs could
361 contribute to the formation of both the autophagosome (Hailey *et al*, 2010; Hamasaki
362 *et al*, 2013) and the mitophagosome (Yang & Yang, 2013). As postmitotic, long-lived
363 cells with complex morphologies, neurons require more careful regulation of

364 mitophagy than other cell types (Evans & Holzbaur, 2019), but the mechanisms that
365 regulate this process in neurons are still poorly understood (Evans & Holzbaur, 2020).

366
367 MERCs mediated by *pdzd8* may limit the rate of mitophagy, analogous to the protective
368 role that mitochondrial fusion is thought to play during starvation-induced autophagy
369 (Gomes *et al*, 2011; Rambold *et al*, 2011; Rana *et al*, 2017; Twig *et al*, 2008). Recently
370 it was reported that increasing several different types of MERC proteins can slow toxin
371 induced mitophagy in non-neuronal cultured cells (McLelland & Fon, 2018; McLelland
372 *et al*, 2018). There is also some recent evidence that MERCs might be directly involved
373 in regulation of mitophagy in neurons (Puri *et al*, 2019). *Pdzd8* has also been found to
374 mediate contacts between the ER and lysosomes (Guillen-Samander *et al*, 2019) and
375 three way contacts between the ER, mitochondria and late endosomes (Elbaz-Alon *et al*,
376 2020; Shirane *et al*, 2020). This may indicate that *Pdzd8*, like other MERC proteins,
377 can play multiple roles at different organelle contacts, or that *Pdzd8* is functional at
378 three-way contacts between the ER, lysosomes and mitochondria (Wong *et al*, 2019).
379 *Pdzd8* function at any of these contact sites could potentially alter mitophagy and future
380 work should explore the mechanisms behind our observed increases in mitophagy.

381
382 While our data suggests that increased mitophagy contributes to the protective effects
383 of the loss of *pdzd8*-mediated MERCs, we have not ruled out contributions from the
384 other specialized functional and signalling roles of MERCs in metabolic regulation,
385 lipid biogenesis and calcium handling (Lee *et al*, 2018a; Rowland & Voeltz, 2012). The
386 increased mitophagy we detected in aged neurons expressing *pdzd8*-RNAi correlates
387 with improved survival and aged locomotor activity, suggesting that reducing the extent
388 of *pdzd8*-mediated MERCs may facilitate more efficient mitophagy and removal of
389 damaged mitochondria from aging neurons. We propose that reducing *pdzd8*-mediated
390 MERCs may be protective in aging neurons by allowing more efficient turnover of
391 mitochondria as damage accumulates with age. As regulators of mitophagy,
392 manipulating MERCs may provide an avenue for enhanced mitochondrial quality
393 control to help promote healthy aging of neurons.

394 **Methods**

395 **Husbandry**

396 Flies were raised under standard conditions at 25°C on food containing agar, cornmeal,
397 molasses, malt extract, soya powder, propionic acid, nipagin and yeast in a 12 h:12 h
398 light:dark cycle.

399

400 **Genetics**

401 *Drosophila* lines were obtained as indicated in Table 1, or generated as described
402 below. All mutant lines used in this study were backcrossed to an isogenic *w*¹¹¹⁸ strain
403 (RRID:BDSC_6326), for 4-6 generations before use. For all integration events,
404 multiple independent lines were initially isolated, verified by PCR and assessed for
405 consistent effects before selecting a single line of each integration site for further study.
406 Wherever possible inert UAS lines such as UAS-*LacZ*-RNAi and UAS-mitoCherry are
407 used as dilution controls to ensure equal numbers of UAS constructs in control and
408 experimental conditions. Unless otherwise stated male flies were used in all
409 experiments.

410

411 **New lines**

412 **SPLICS**

413 The SPLICSs construct with an 8-10 nm range from Tito Cali & Marisa Brini (Cieri *et*
414 *al*, 2017), was amplified from pSYC-SPLICSs-P2A using
415 (TAAGCAGCGGCCGCTGATTTAGGTGACACTATAG) and T7 forward primer
416 (TAATACGACTCACTATAGGG) and cloned into pUAST-AttB between NotI and
417 XbaI sites. Flies were injected by BestGene to insert into attP16 (II) and attP2 (8622,
418 III) and the attP16 site gave better signal and so was used in this work. The number of
419 puncta produced in the axon bundles driven by nSyb-GAL4 varied with a consistently
420 more puncta than in the central axons bundles in the peripheral bundles (Fig S2B).

421 **pdzd8-HA**

422 pdzd8-HA was synthesized by Genewiz based on the cDNA Genbank sequence
423 LD34222 (AY118553.1) (Sayers *et al*, 2020), and cloned into pUAST.attB between
424 EcoRI and XbaI. The University of Cambridge Department of Genetics Fly Facility
425 generated lines by injection of this construct into the attP40 landing site.

426

427 **Climbing**

428 The startle induced negative geotaxis (climbing/locomotor) assay was performed as
429 described previously (Andreazza *et al*, 2019). Briefly, a maximum of 23 males were
430 placed into the first tube of a countercurrent apparatus, tapped to the bottom, and given
431 10 s to climb 10 cm. This procedure was repeated five times (five tubes), and the
432 number of flies that has remained into each tube counted and the climbing performance
433 expressed as a climbing index (Greene *et al*, 2003). The same flies were aged and
434 assayed again on the indicated days post-eclosion.

435

436 **Lifespan**

437 For lifespan experiments groups of approximately 20-25 males were collected with
438 minimal time under anesthesia (CO₂), placed into separate vials with food and
439 maintained at 25°C. Flies were transferred into fresh vials every 2-3 days, and the
440 number of dead flies were recorded. Percent survival was calculated using
441 <https://flies.shinyapps.io/Rflies/>. To assess lifespan in a diet with restricted nutrients
442 flies were raised in standard conditions then transferred to tubes containing food made
443 from 5 % sucrose and 1 % agar and flipped every 2-3 days. Lifespans in the presence
444 of mitochondrial toxins and hydrogen peroxide were also performed on food made from
445 5 % sucrose and 1 % agar cooled to less than 50°C before adding toxin at 1:1000.
446 Rotenone (Sigma R8875) was dissolved in DMSO (1 mM final concentration) and
447 antimycin A (Sigma A8674) (4 µg/mL final concentration) dissolved in 70 % ethanol.
448 Flies in toxin assays were starved for 5 h before being placed on food containing toxins.
449 Flies in rotenone assays were monitored twice a day and flipped every two days. Flies
450 in antimycin A assays were monitored three times a day and flipped every two days.

451

452 **Fluorescence Microscopy**

453 Imaging of larval axons was performed as described in (Wang & Schwarz, 2009) with
454 the following variations: wandering third instar larvae were pinned at each end dorsal
455 side up to a reusable Sylgard (Sigma 761028) coated slide using pins (Fine Science
456 Tools FST26002-10) cut to ~5 mm and bent at 90°. The larvae were cut along the dorsal
457 midline using micro-dissection scissors. Internal organs were removed with forceps
458 without disturbing the ventral ganglion and motor neurons. Larvae were then covered
459 in dissection solution (Godena *et al*, 2014). The cuticle was then pulled back with four

460 additional pins. The anterior pin was adjusted to ensure axons are taut and as flat as
461 possible for optimal image quality.

462

463 Movies were taken using a Nikon E800 microscope with a 60x water immersion lens
464 (NA 1.0 Nikon Fluor WD 2.0) and an LED light source driven by Micromanager 1.4.22
465 Freeware (Edelstein *et al*, 2014). A CMOS camera (01-OPTIMOS-F-M-16-C) was
466 used to record 100 frames at a rate of 1 frame per 5 s (8 min 20 s total). Axons were
467 imaged within 200 μm of the ventral ganglion in the proximal portion of the axons and
468 no longer than 1 h after dissection. Movies were converted into kymographs using Fiji
469 (Schindelin *et al*, 2012) and mitochondrial motility quantified manually with the
470 experimenter blinded to the condition.

471

472 For SPLICS imaging in axon bundles at least three ROI 50 μm x 12 μm were quantified
473 per animal and averages for each larva were plotted. For SPLICS quantification puncta
474 intensity varied considerably so blinded manual counting was used.

475

476 To image NMJs larvae were dissected as described above and fixed for 20 min in 4 %
477 formaldehyde in PBS. After blocking for 1 h in 1 % BSA/0.3 % Triton X-100/PBS
478 solution, anti-HRP was added at 1:500 and samples agitated gently overnight at 4°C.
479 After three washes in 0.3 % Triton X-100/PBS at room temperature, samples were
480 incubated with Alexa Fluor 594 at 1:500 for 1 h in 1% BSA/0.3% Triton X-100/PBS
481 solution. Samples were then washed in 3x in PBS before being mounted in Prolong
482 Diamond. NMJs were imaged on a Nikon Eclipse TiE inverted microscope with
483 appropriate lasers using an Andor Dragonfly 500 confocal spinning disk system, using
484 an iXon Ultra 888 EMCCD camera (Andor), coupled with Fusion software
485 (Andor) using a 60x NA 1.49 objective. NMJs on muscle 4 from segments A3 and A4
486 (NMJs on these segments are the same size (Nijhof *et al*, 2016)) were captured in Z
487 stacks with 0.3 μm step size and analysed using Imaris (x64 9.2.0) to determine NMJ
488 volume, mitochondrial volume and mitochondrial number.

489

490 For MitoQC imaging, samples were fixed for 30 min in 4 % formaldehyde (16 % VWR
491 100503) diluted in pH 7.0 PBS. Adult brains were mounted in Prolong Diamond
492 Antifade Mountant (Thermofisher P36961) using spacers and imaged on a Carl Zeiss
493 LSM880 confocal laser-scanning system on an Axio Observer Z1 microscope (Carl
494 Zeiss), coupled with ZEN software (Carl Zeiss) using a 100x Plan-APOCHROMAT
495 /1.4 oil DIC objective. Images are shown in false colour with magenta puncta
496 representing mCherry signal indicating where the reporter is in an acidic environment
497 of a lysosome and the GFP has been quenched (Allen *et al*, 2013).

498

499 Imaging of wings was performed as described in Vagnoni & Bullock (Vagnoni &
500 Bullock, 2016). Briefly, flies were anaesthetized with CO₂, and immobilised with their
501 wings outstretched on a cover glass with a fine layer of Halocarbon oil (VWR). A
502 second cover glass was then added on top of the fly to stabilize the sample. Live
503 imaging in the wing nerves was performed using a Nikon spinning disk system
504 essentially as described previously (Morotz *et al*, 2019). The mitoQC puncta were
505 annotated with Fiji using the Cell Counter plugin and quantified with the experimenter
506 blinded to the genotype.

507

508 For imaging of the larval epidermal cells, the larvae were dissected as described above
509 but the nervous system was also removed before fixation. The samples were washed in

510 PBS and the muscles were then removed (Tenenbaum & Gavis, 2016). The dissected
511 filets were mounted in Prolong Diamond Antifade Mountant using No. 1.5H High
512 Precision Deckglaser cover slips and placed under a weight for 24 h. N-SIM (Nikon
513 Structured Illumination Microscopy) imaging was performed on a Nikon Ti Eclipse
514 with an Andor DU-897 X-5835 camera and SR Apo TIRF 100x (NA1.5) objective run
515 using NIS-Elements 4.60. Images were analysed in Fiji (Schindelin *et al*, 2012) using
516 the Coloc2 plugin.

517

518 **Transmission Electron Microscopy**

519 Transmission electron microscopy (TEM) was performed at Cambridge Advanced
520 Imaging Center (CAIC). Brains of 2 day old adult flies were fixed in 2 %
521 glutaraldehyde/ 2 % formaldehyde in 0.1 M sodium cacodylate buffer pH 7.4
522 containing 2 mM CaCl₂ and 0.1 % Tween20 (based on method described in (Celardo
523 *et al*, 2016)), overnight at 4°C. Samples were then washed 5x with 0.1 M sodium
524 cacodylate buffer and then treated with osmium for 2 days at 4°C (1 % OsO₄, 1.5 %
525 potassium ferricyanide, 0.1 M sodium cacodylate buffer pH 7.4). Samples were then
526 washed 5x in distilled water and treated with 0.1 % aqueous thiocarbohydrazide for 20
527 min in the dark at room temperature. Samples were washed another 5x in distilled water
528 then treated with osmium a second time for 1 h at room (2 % OsO₄ in distilled water).
529 Samples were then washed another 5x in distilled water before being treated with uranyl
530 acetate bulk stain for 3 days at 4°C (2 % uranyl acetate in 0.05 M maleate buffer pH
531 5.5). After a final 5x wash in distilled water, samples were dehydrated in 50/70/95/100
532 % ethanol, 3x in each for at least 5 min each. Dehydration was completed by two further
533 treatments with 100 % dry ethanol, 2x in 100 % dry acetone and 3x in dry acetonitrile
534 for at least 5 min each. Quetol resin mix (12 g Quetol 651, 15.7 g NSA, 5.7 g MNA,
535 0.5 g benzyltrimethylamine) made with an equal volume of 100 % dry acetonitrile and
536 samples placed in this mix for 2 h at room temperature. Samples were then incubated
537 in pure Quetol resin mix for 5 days, exchanging the samples to fresh resin mix each
538 day. After 5 days the brains were embedded in coffin moulds and cured at 60°C for at
539 least 48 h. Ultrathin sections were cut on a Leica Ultracut E at 70 nm thickness. Sections
540 were mounted on 400 mesh bare copper grids and viewed in a FEI Tecnai G20 electron
541 microscope run at 200 keV using a 20 µm objective aperture. Quantification of the
542 percentage of clearly identifiable mitochondria in contact with ER was performed
543 manually as described in (Celardo *et al*, 2016) and the experimenter was blinded to the
544 genotype.

545

546

547 **qPCR**

548 Five female wandering third instar larvae per sample were washed briefly in 1xPBS,
549 placed in RNase free tubes and frozen on dry ice. Larvae were homogenized in Trizol
550 and RNA isolated by phenol:chloroform extraction and isopropanol precipitation.
551 DNase treatment using Invitrogen TURBO DNA-free rigorous procedure was
552 performed before measuring RNA concentration with a Qubit[®] RNA HS Assay Kit
553 (Molecular Probes, Life Technologies). Reverse transcription reactions used 1.32 µg of
554 RNA using SuperScript III Reverse Transcriptase (Invitrogen) with Oligo(dT)23VN as
555 per manufacturer's instructions. The resulting cDNA was used for qPCRs using
556 PowerUp SybrGreen (Applied Biosystems A25742). Primers for pdzd8 were PDZD8-
557 F TTCTGTTTGGCTTCTCCTGG, PDZD8-R TTGAGGAAGTGC GACTGATC
558 designed using RealTime qPCR Assay Entry (idtdna.com). *aTub84B* (Fwd:
559 TGGGCCCGTCTGGACCACAA, Rev: TCGCCGTCACCGGAGTCCAT), *vkg* (Fwd:

560 CGAGGATGTTACCCAGAGATC, Rev: TCGTCCCTTGATTCCTTTG), *COX8*
 561 (Fwd: CAGAGCCGTTGCCAGTC, Rev: CTTGTCGCCCTTGTAGTCC), and *Rpl32*
 562 (Fwd: AAACGCGGTTCTGCATGAG, Rev: GCCGCTTCAAGGGACAGTATCTG)
 563 were used as housekeeping genes with their values combined to compare knockdown
 564 to the geometric mean (Taylor *et al*, 2019).
 565

566 ATP

567 ATP levels were measured in two- and twenty-day-old fly heads with 40 flies per
 568 genotype and three biological replicates. The ATP levels were measured as described
 569 in Tufi, Gleeson *et al*. (Tufi *et al*, 2019) with minor modifications. Briefly, heads were
 570 homogenized in 6 M guanidine-Tris/EDTA extraction buffer and subjected to rapid
 571 freezing in liquid nitrogen. Luminescence produced from homogenates mixed with the
 572 CellTiter-Glo Luminescent Cell Viability Assay (Promega) was measured with a
 573 SpectraMax Gemini XPS luminometer (Molecular Devices) and normalized to total
 574 protein, quantified using the Pierce BCA method (Thermo Fisher Scientific).
 575

576 Quantification & Statistical analysis

577 Statistical analyses were performed using GraphPad Prism 8 software. Data are
 578 reported as mean \pm 95 % CI unless otherwise stated in figure legends. Climbing was
 579 assessed using a Kruskal-Wallis non-parametric test with Dunn's post-hoc correction
 580 for multiple comparisons. Lifespans were compared using log-rank Mantel-Cox tests.
 581 Number of flies and p-values are reported in the figure legends.
 582

583 Mitochondrial transport was analysed using ordinary one-way ANOVA and Holm-
 584 Sidak's multiple comparison. ATP measurements were analysed by two-tailed t-test.
 585 Values are not significantly different to controls unless otherwise stated.
 586

587 SCoPe (<http://scope.aertslab.org/>) was used to visualize transcriptome data from the
 588 unfiltered adult fly brain dataset (Davie *et al*, 2018).
 589

590 **Table S1: Fly Lines**

Label	Line	Source	Reference	RRID
nSyb	P{nSyb-GAL4.S}3 III	BL51635	Bloomington	BDSC_51635
OK371	P{GawB}VGlut ^{OK371}	BL26160	(Mahr & Aberle, 2006)	BDSC_26160
da	da-GAL4 III	Strutt Lab	Bloomington	BDSC_55850
Act	P{Act5C-GAL4}25FO1	BL4414	Bloomington	BDSC_4414
CCAP	P{CCAP-GAL4}16 (II)	BL25685	(Park <i>et al</i> , 2003)	BDSC_25685
tether	UAS-tether	Scorrano Lab	(Basso <i>et al</i> , 2018) tether high (TH) construct from (Csordas <i>et al</i> , 2006)	
LacZ-RNAi	P{GD936}v51446 II UAS-LacZ-RNAi	VDRC (Strutt Lab)	(Dietzl <i>et al</i> , 2007)	FlyBase_FBst_0469426
pdzd8-RNAi	NIG 10362R-2 UAS-pdzd8-RNAi III	This study	The fly stock was obtained from NIG-Fly Stock Center.	
mitoGFP (II)	P{UAS-mito-HA-GFP.AP}2	BL8442	(Pilling <i>et al</i> , 2006)	BDSC_8442
mitoGFP (III)	P{UAS-mito-HA-GFP.AP}3, e1	BL8443	(Pilling <i>et al</i> , 2006)	BDSC_8443

mitoCherry	P{UAS-mCherry.mito.OMM}(II I)	Alessio Vagnoni	(Vagnoni <i>et al</i> , 2016)	BDSC_66533
ER-Tom	PBac{20XUAS-tdTomato-Sec61β}VK00037 (II)	BL64746	(Summerville <i>et al</i> , 2016)	BDSC_64746
SPLICS	SPLICSs#4	This study	Construct from (Cieri <i>et al</i> , 2017)	
mitoQC	UAS-mitoQC (II)	Whitworth Lab	(Lee <i>et al</i> , 2018b)	
Aβ	P{UAS-Aβ.Arctic}	Isabel Palacios	(Crowther <i>et al</i> , 2005) FBal0248069	
<i>pdzd8</i> -HA	P{UAS- <i>pdzd8</i> -HA}(attP40)	This study	University of Cambridge Department of Genetics Fly Facility	

591

592 **Table S2: Genotypes in figures**

Figure 1 (B, C)	<i>LacZ</i> -RNAi/+; nSyb/+ <i>pdzd8</i> -RNAi/nSyb
Figure 1 (D, E)	SPLICS/ <i>LacZ</i> -RNAi; nSyb/+ SPLICS/+; <i>pdzd8</i> -RNAi/nSyb
Figure 1 (F, G)	ER-Tom, mitoGFP/ <i>LacZ</i> -RNAi; da/+ ER-tom, mitoGFP/+; da/ <i>pdzd8</i> -RNAi
Figure 2 (A, C)	<i>LacZ</i> -RNAi/+; nSyb/+ <i>pdzd8</i> -RNAi/nSyb
Figure 2 (B, D)	<i>LacZ</i> -RNAi/+; nSyb/+ tether/nSyb
Figure 3 (A-D)	<i>LacZ</i> -RNAi/+; nSyb/+ <i>pdzd8</i> -RNAi/nSyb
Figure 4 (A-E)	CCAP/+; mitoGFP/+ CCAP/+; <i>pdzd8</i> -RNAi/mitoGFP CCAP/+; tether/mitoGFP
Figure 4 (F-I)	OK371, mitoGFP/ <i>LacZ</i> -RNAi OK371, mitoGFP/+; <i>pdzd8</i> -RNAi/+
Figure 5 (A-F)	mitoQC/ <i>LacZ</i> -RNAi; nSyb/+ mitoQC/+; nSyb/ <i>pdzd8</i> -RNAi
Figure 6 (A-B)	<i>LacZ</i> -RNAi/SPLICS; nSyb/+ Aβ/SPLICS; nSyb/+
Figure 6 (C)	<i>LacZ</i> -RNAi/+; nSyb/+ <i>LacZ</i> -RNAi/Aβ; nSyb/+ <i>LacZ</i> -RNAi/Aβ; <i>pdzd8</i> -RNAi/nSyb
Figure S1 (C)	Act/ <i>LacZ</i> -RNAi (females) Act/+; <i>pdzd8</i> -RNAi/+ (females)
Figure S1 (D)	ER-Tom, mitoGFP/ <i>LacZ</i> -RNAi, nSyb/+ ER-Tom, mitoGFP/+, nSyb/ <i>pdzd8</i> -RNAi
Figure S1 (E)	ER-Tom, mitoGFP/ <i>LacZ</i> -RNAi; da/+ ER-tom, mitoGFP/+; da/ <i>pdzd8</i> -RNAi
Figure S2 (B)	SPLICSs/+; nSyb-GAL4/+
Figure S2 (D)	SPLICS/ <i>LacZ</i> -RNAi; nSyb/+ SPLICS/+; tether/nSyb

Figure S3 (A)	OK371/ <i>LacZ</i> -RNAi
	OK371/+; <i>pdzd8</i> -RNAi/+
Figure S3 (B)	<i>LacZ</i> -RNAi/+; mitoCherry/nSyb,
	<i>LacZ</i> -RNAi/ <i>pdzd8</i> -HA; nSyb/+
	<i>pdzd8</i> -HA/+; <i>pdzd8</i> -RNAi/nSyb
	tether/nSyb

593

594 **Figure Legends**

595 **Figure 1.**

596 **Expression of *pdzd8*-RNAi reduces mitochondria-ER contacts.** (A) Domain
597 organisation of *Drosophila pdzd8* (CG10362) compared to mouse *Pdzd8* showing
598 percentage identities of conserved domains based on Clustal Omega alignments.
599 Overall percentage identity of the amino acid sequences is 21 %. SMP (Synaptotagmin-
600 like mitochondrial lipid-binding proteins) 33 % identical, PDZ (PSD95/DLG/ZO-1) 36
601 % identical, C1 (C1 protein kinase C conserved region 1 also known as Zn finger
602 phorbol-ester/DAG-type signature) 47 % identical; TM: predicted transmembrane
603 domain, CC: coil-coil domain. (B) Electron microscopy images of 2-day-old adult
604 brains showing representative images of ER, mitochondria and MERCs in soma from
605 nSyb>*LacZ*-RNAi and nSyb>*pdzd8*-RNAi flies. Scale bar 500 nm. Mitochondria
606 without identifiable ER contacts marked with magenta *, mitochondria forming ER
607 contact marked with yellow * with yellow arrow indicating contact location, organelles
608 that did not contain clear cristae are marked with a cyan * and were excluded from the
609 analysis (C) Percentage of mitochondria in contact with the ER from controls and pan-
610 neuronal nSyb>*pdzd8*-RNAi flies quantified from EM images of 2-day-old adult brains.
611 N = 3 brains per genotype. (D) SPLICS puncta indicating MERCs in axon bundles of
612 larval neurons from controls and nSyb>*pdzd8*-RNAi flies. Quantified puncta
613 highlighted with V. (E) Quantification of SPLICS puncta in C. n = 11 animals per
614 genotype, p=0.0198, unpaired t-test with Welch's Correction, scale bar 5 μ m. (F)
615 Representative binarized SIM images of ER (green) and mitochondria (purple) in larval
616 epidermal cells from controls and da>*pdzd8*-RNAi flies. Scale bar 500 nm. (G)
617 Quantification of colocalization of ER and mitochondria using Mander's Correlations
618 compared using an unpaired t-test with Welch's Correction. p=0.012.

619

620 **Figure 2.**

621 **Lifespan and locomotor activity changes in aged flies with pan-neuronal (nSyb)
622 driven alterations in tethering.** (A, B) Locomotor activity of flies was assessed during
623 aging by negative geotaxis climbing assays on the indicated days. n>50 flies per
624 genotype. Flies expressing (A) *pdzd8*-RNAi or (B) synthetic tether were compared to
625 *LacZ*-RNAi controls. (C, D) Lifespans in standard growth conditions and food. (C)
626 Flies expressing *pdzd8*-RNAi were compared to *LacZ*-RNAi controls. n = 97, 108 per
627 genotype, median survival 44 vs 52 days, p<0.0001. (D) Flies expressing the synthetic
628 tether were compared to *LacZ*-RNAi controls. n = 74, 85 per genotype, median survival
629 52 vs 33 days, p<0.0001.

630

631 **Figure 3.**

632 **Knockdown of *pdzd8* protects flies against mitochondrial toxins.** Flies expressing
633 pan-neuronal nSyb>*pdzd8*-RNAi were compared to nSyb>*LacZ*-RNAi controls when
634 aged on a restricted diet of food containing 1 % agar with 5 % sucrose. (A) Lifespan

635 with dietary restriction alone. N = 62 vs 64, median survival 20 days vs 19 days,
636 difference ns. **(B)** Lifespan with addition of 5 % hydrogen peroxide. Median survival:
637 63 h vs 74 h, n = 67, 74, p=0.0002. **(C)** Lifespan with addition of 1 mM rotenone.
638 Median survival: 11 days vs 20 days, n = 66, 57, p<0.0001. **(D)** Lifespan with addition
639 of 5 µg/mL antimycin A. Median survival: 74 h vs 68 h, n = 72, 68, p=0.0002.
640

641 **Figure 4.**

642 **Knockdown of *pdzd8* in larval neurons causes minor defects while increasing**
643 **MERCs is detrimental to in axonal mitochondria size and motility.** **(A)**

644 Representative images of mitochondrial morphology and distribution in larval axons.
645 Mitochondria were detected using CCAP>mitoGFP in controls and *pdzd8*-RNAi
646 expressing larvae. Scale bar 5 µm. **(B,C)** Mitochondrial length **(B)**, and mitochondrial
647 density **(C)** in the larval axons shown in A were analysed using ordinary one-way
648 ANOVA and Holm-Sidak's multiple comparisons. n = 10, 10, 13 animals, data points
649 represent different axons, all differences ns. **(D)** Representative kymographs showing
650 motility of CCAP>mitoGFP signal in controls and *pdzd8*-RNAi expressing larvae.
651 Stationary mitochondria appear as vertical lines, moving mitochondria form diagonal
652 lines in anterograde or retrograde directions, t = 500s **(E)** Quantification of
653 mitochondrial transport shown in D, analysed using ordinary one-way ANOVA and
654 Holm-Sidak's multiple comparisons, n = 14-25, p<0.0001. **(F)** Representative images
655 of NMJs and mitochondria of controls and *pdzd8*-RNAi labelled using
656 OK371>mitoGFP. magenta = mitoGFP, green = anti-HRP (neuronal membrane), scale
657 bar 10 µm. **(G-I)** Quantifications of NMJ volume, p=0.0036, **(G)**, mitochondrial
658 volume, p=0.002 **(H)** and mitochondrial density **(I)** were compared using an unpaired
659 t-test with Welch's Correction.
660

661 **Figure 5.**

662 **Pan-neuronal *pdzd8*-RNAi increases mitophagy during aging.** **(A)** Representative
663 images of MitoQC signal in wandering L3 larval ventral ganglia. magenta = mCherry,
664 green = GFP, images show a single plane of a Z-stack, scale bar 2 µm. **(B)**
665 Quantification of MitoQC puncta shown in **(A)**, n = 9, differences ns. **(C)**
666 Representative images of MitoQC signal in adult brains in two- and ten-day old flies,
667 magenta = mCherry, green = GFP, scale bar 5 µm, image shows a single plane of a Z-
668 stack. **(D)** Quantification of MitoQC signal in adult brains and compared using an
669 unpaired t-test with Welch's correction (n = 7-9, p=0.0072) **(E)** the Representative
670 images of MitoQC signal in 14 day-old fly wings. Only mCherry signal (magenta) is
671 shown for clarity, white outlines edges of wing nerves, scale bar 5 µm. **(F)**
672 Quantification of MitoQC signal in aged fly wings at 2, 14 and 30 days post eclosion
673 using a one-way ANOVA with Holm-Sidak's multiple comparisons. n (2 days) = 33,
674 26 (14 days) = 24, 31, (30 days) = 32, 12.
675

676 **Figure 6.**

677 **Reducing *pdzd8*-mediated MERCs rescues the locomotor defects in an**
678 **Alzheimer's disease *Drosophila* model.** **(A)** CCAP>SPLICS signal in larval axons
679 expressing Aβ₄₂ compared to controls. Quantified puncta highlighted with V. **(B)**
680 Quantification of SPLICS signal in **(A)** using an unpaired t-test with Welch's
681 correction, n = 6, p=0.008. **(C)** Aged climbing assay of flies expressing nSyb>Aβ₄₂,
682 n>75 flies were aged and climbed on the indicated days. p <0.001, 0.002, <0.001,
683 <0.001.

684

685 **Figure S1.**

686 **Expression and tissue specificity of *pdzd8*.** (A) Tissue specific expression of *pdzd8*
687 in 7 day old adult males from (Leader *et al*, 2018) FPKM: fragments per kilobase of
688 exon model per million reads mapped providing a normalized estimation of gene
689 expression based on RNA-seq data. (B) SCoPe transcriptome data from the unfiltered
690 adult fly brain dataset. (C) Relative abundance of *pdzd8* transcript in controls compared
691 to Tub>*pdzd8*-RNAi normalised to the relative to geometric mean of housekeeping
692 genes *αTub84B*, *vkg*, *COX8* and *Rpl32*; p=0.0134. (D) Representative SIM images of
693 ER (green) and mitochondria (purple) in L3 larval neurons, scale bar 5 μm. (E)
694 Representative SIM images of ER and mitochondria in larval epidermal cells, scale bar
695 5 μm. (F) Close up inlays (F'-F''') of example MERCs in Figure 1E identified in
696 nSyb>*LacZ*-RNAi brains imaged using electron microscopy. Scale bar 500 nm.

697

698 **Figure S2.**

699 **SPLICS and tether constructs used in this study.** (A) Cartoon of SPLICS targeting
700 and mode of action (Created with BioRender.com). (B) Density of SPLICS puncta in
701 axons is different in different axon bundles, scale bar 5 μm. (C) Cartoon of the synthetic
702 tether construct targeting and mode of action (Created with BioRender.com). (D)
703 nSyb>SPLICS puncta indicating contact sites in larval axons, scale bar 5 μm, n = 9 per
704 genotype.

705

706 **Figure S3.**

707 **Phenotypic characterization of altered tethering in motor neurons.** (A) Motor
708 neuron-specific aged climbing assay showing OK371>*pdzd8*-RNAi compared to *LacZ*-
709 RNAi controls. (B) *pdzd8*-RNAi rescues aged climbing defect resulting from *pdzd8*
710 over expression. (C) nSyb>tether expression resulted in severely deformed NMJs on
711 muscle 4 and made it impossible to distinguish NMJs type 1s and 1b synaptic boutons
712 (therefore mitochondrial density could not be quantified). (D) ATP levels in fly heads
713 analysed showing mean ± standard deviation, n = 3, 40 flies per replicate, compared
714 using a two-tailed t-test, all differences ns.

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731

732 **Conflict of Interests**

733 The authors declare that they have no conflict of interest.

734

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745

746 **Author Contributions**

747 VLH, LM-F, SA, JP, FM and AV designed and/or performed experiments, and
748 analysed the data. VLH wrote the manuscript with input from all authors. AJW, AV
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Figure 1. Expression of *pdzd8*-RNAi reduces mitochondria-ER contacts.

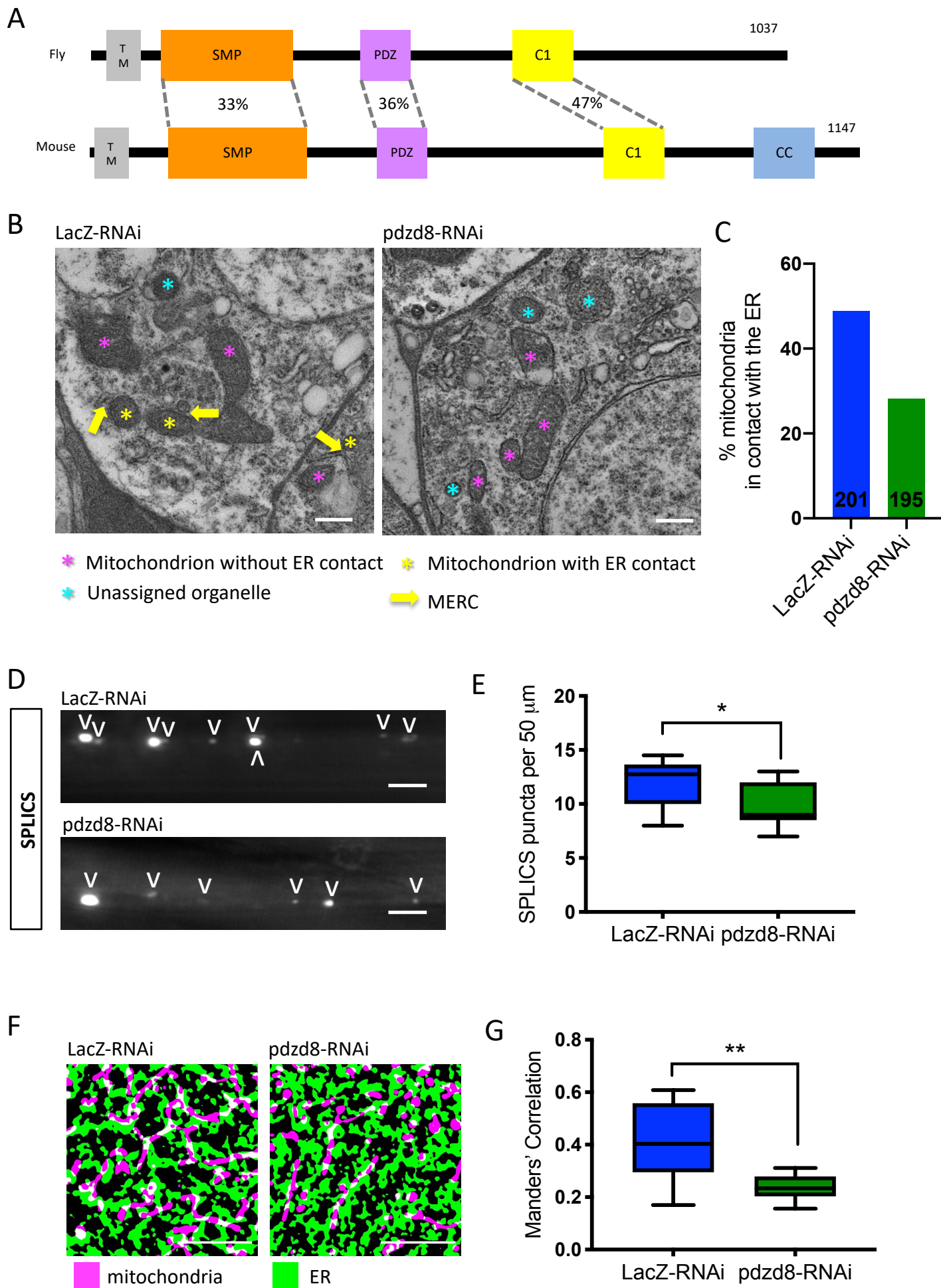


Figure 2. Lifespan and locomotor activity changes in aged flies with pan-neuronal (nSyb) driven alterations in tethering.

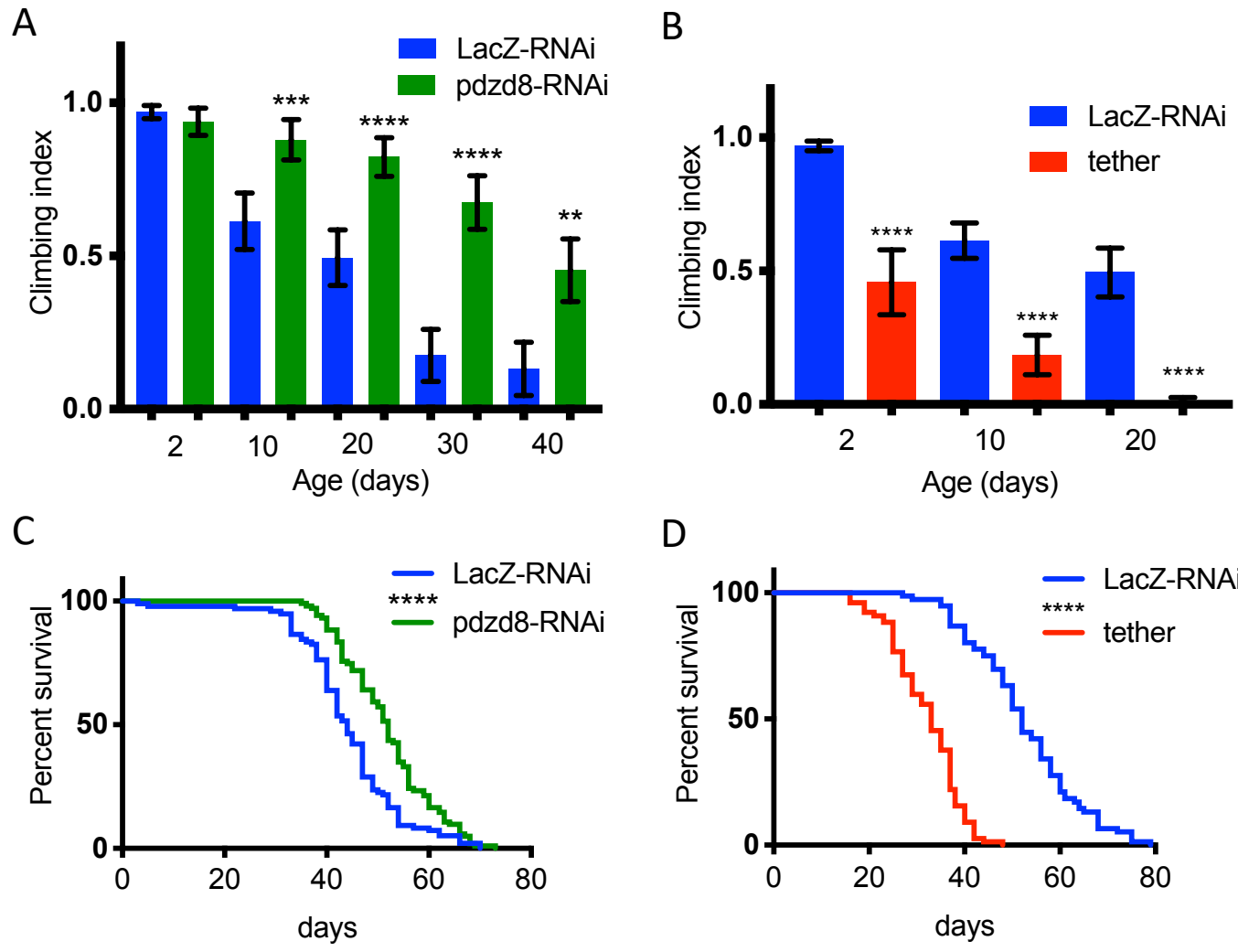


Figure 3. Knockdown of *pdzd8* protects flies against mitochondrial toxins.

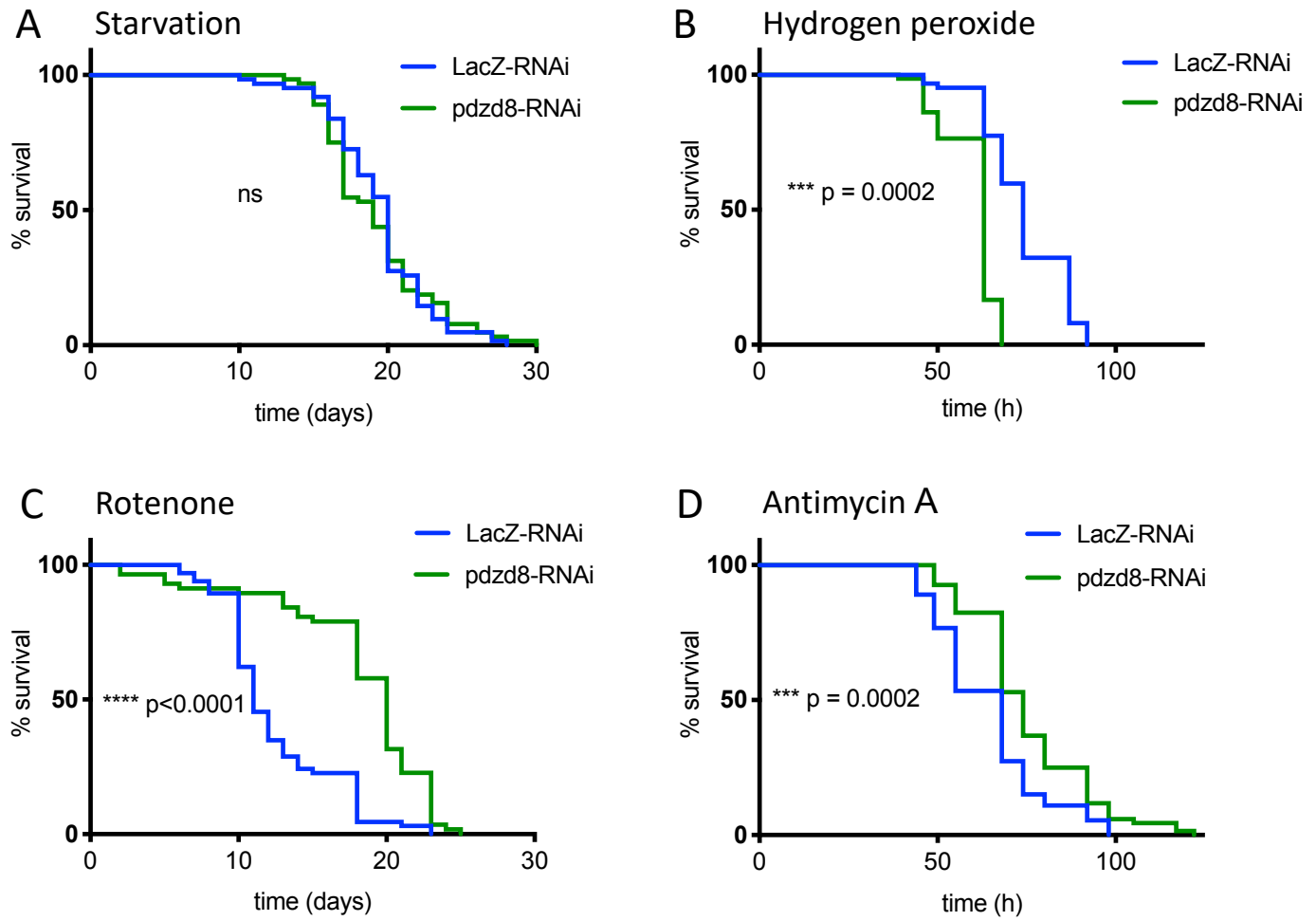


Figure 4. Knockdown of *pdzd8* in larval neurons causes minor defects while increasing MERCs is detrimental to in axonal mitochondria size and motility.

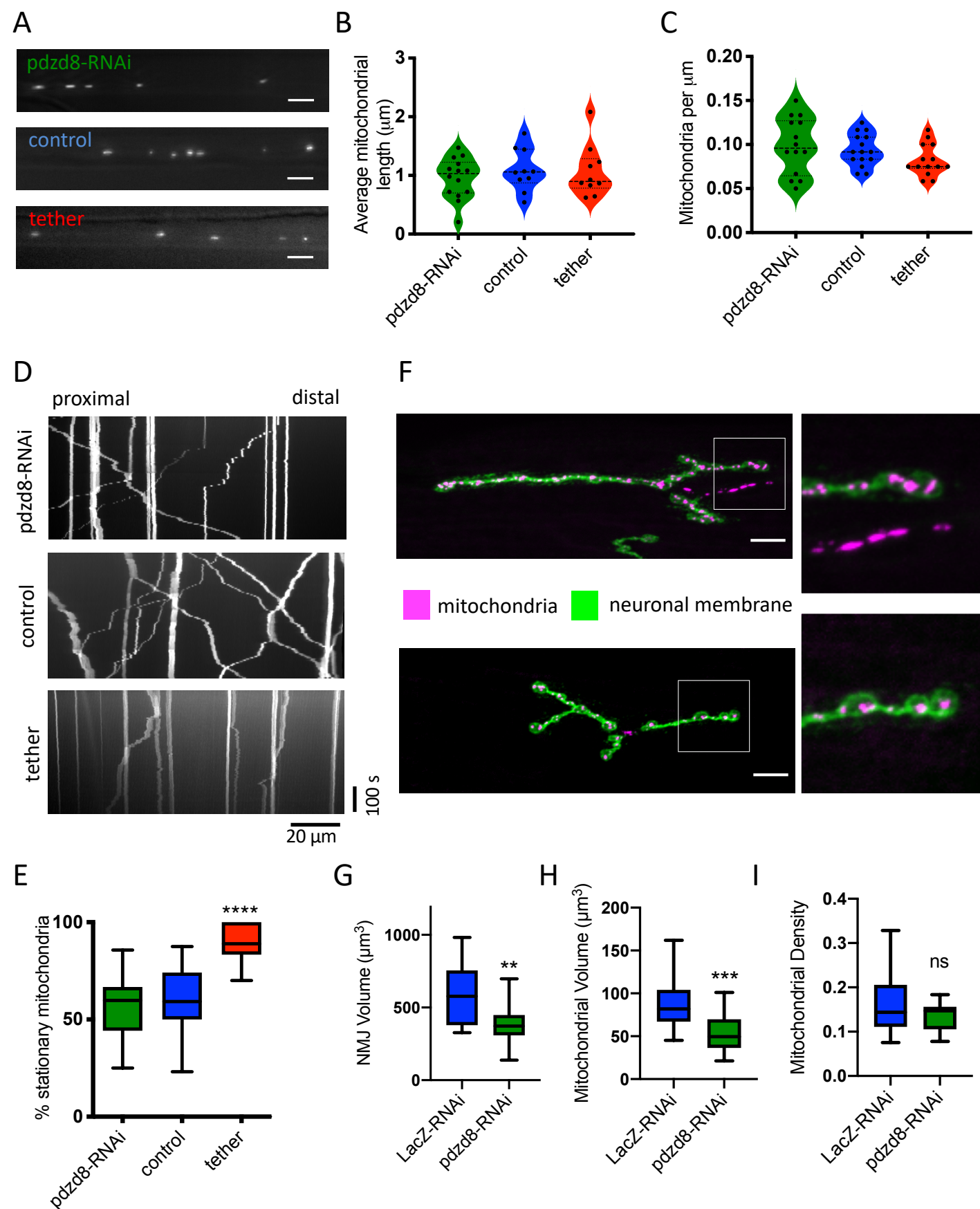


Figure 5. Pan-neuronal *pdzd8*-RNAi increases mitophagy during aging.

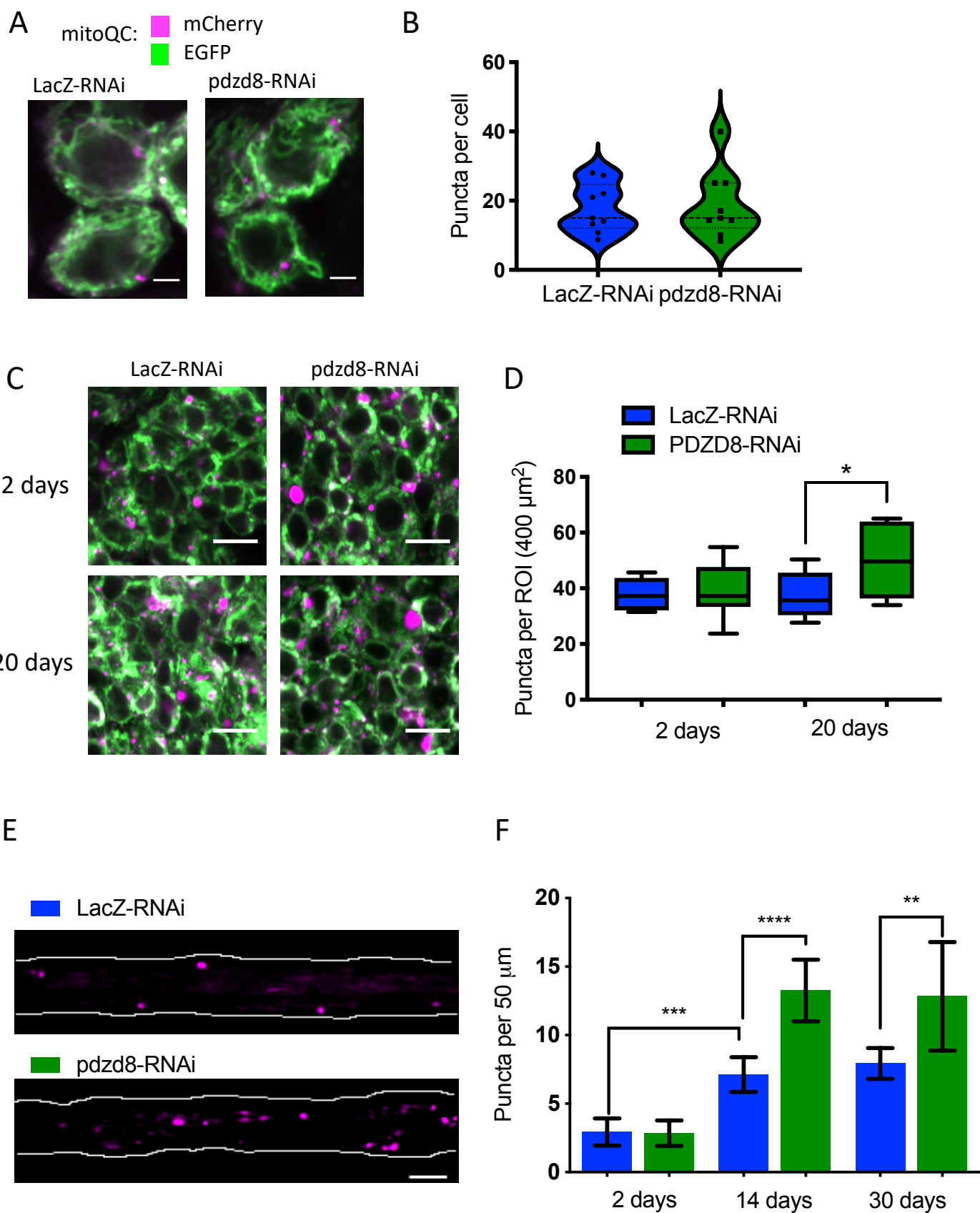


Figure 6. Reducing pdzd8-mediated MERCs rescues the locomotor defects in an Alzheimer's disease *Drosophila* model.

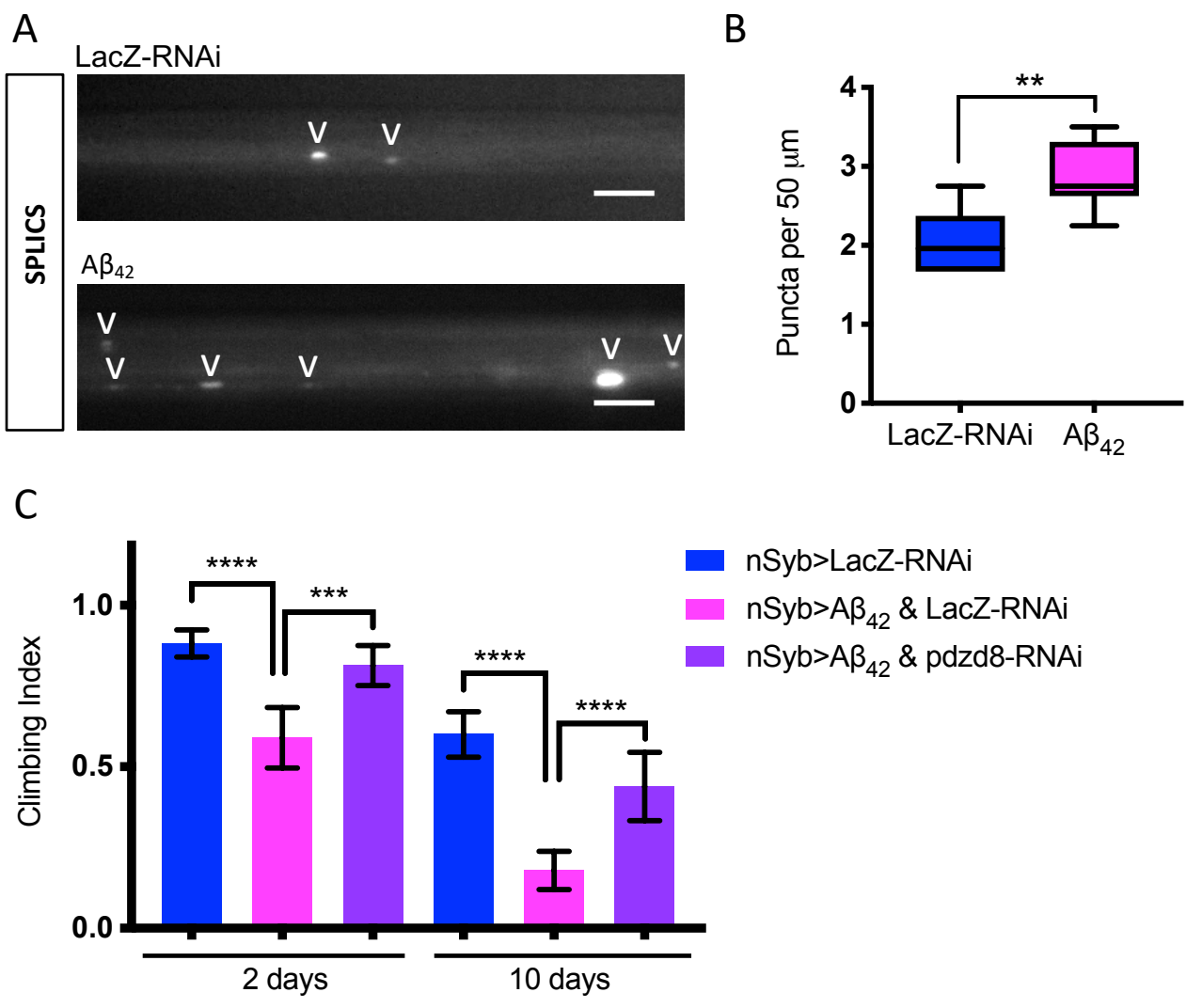


Figure S1. Expression and tissue specificity of pdzd8.

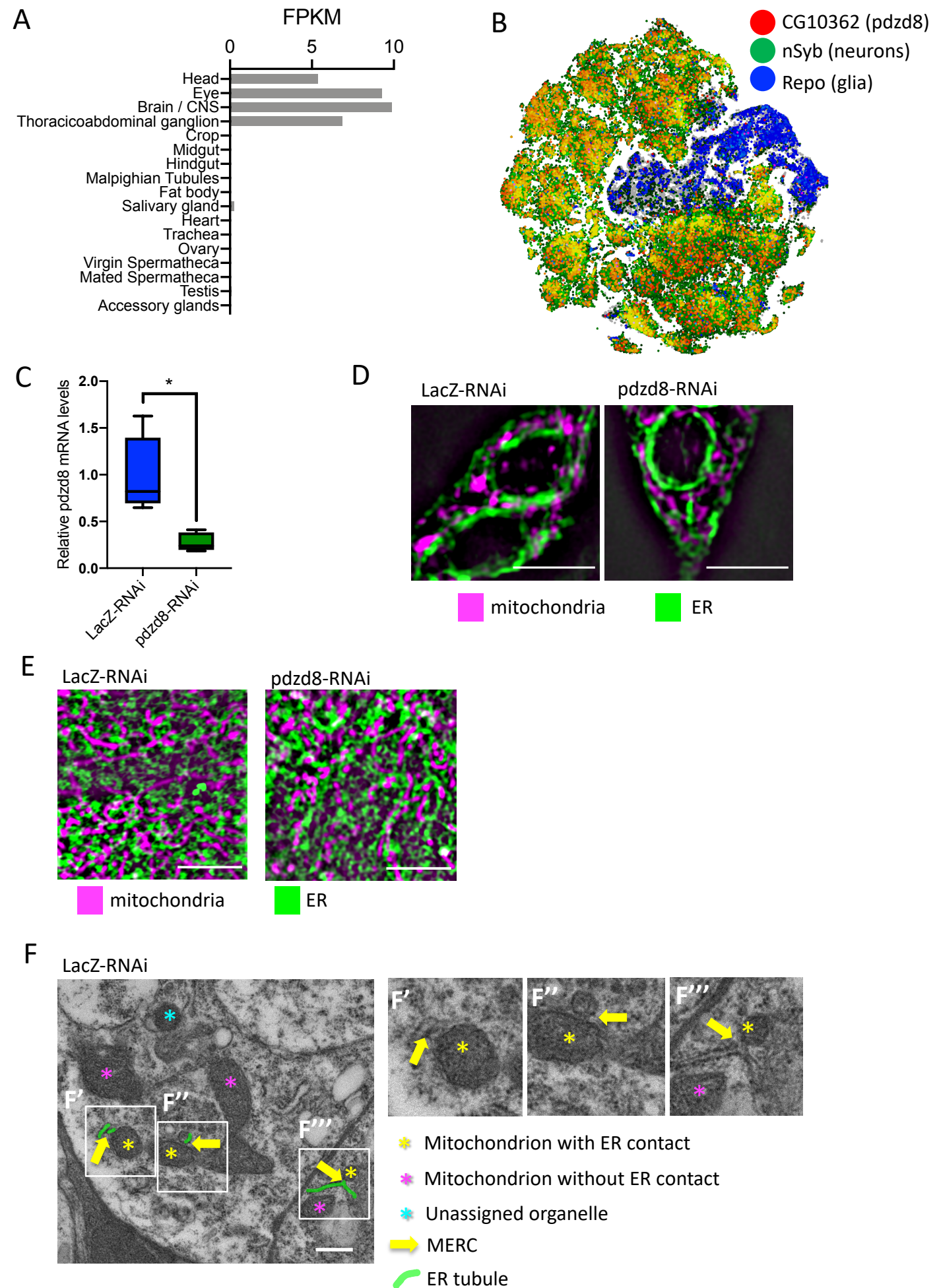


Figure S2. SPLICS and tether constructs used in this study.

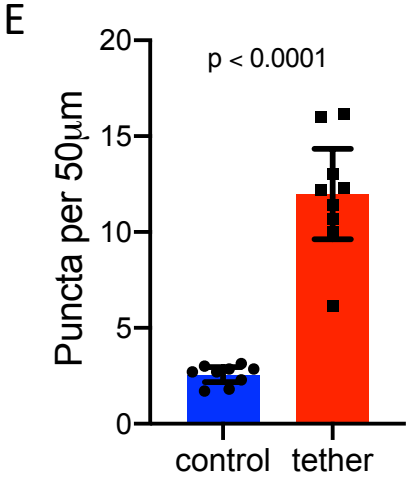
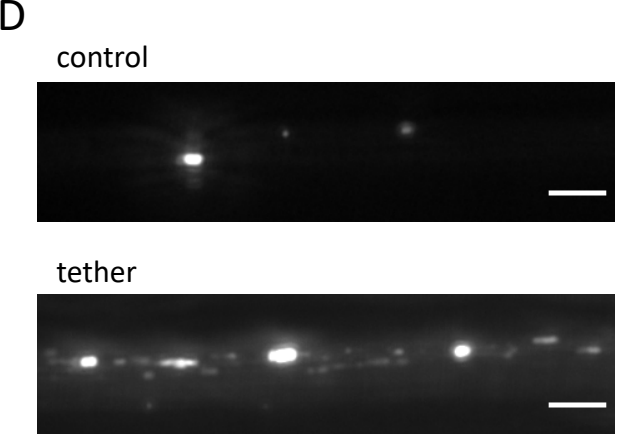
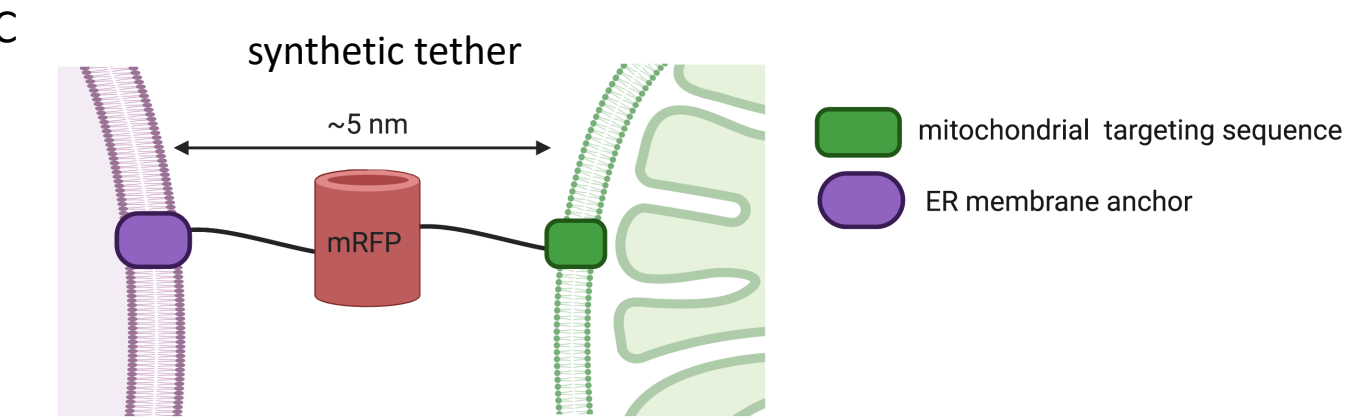
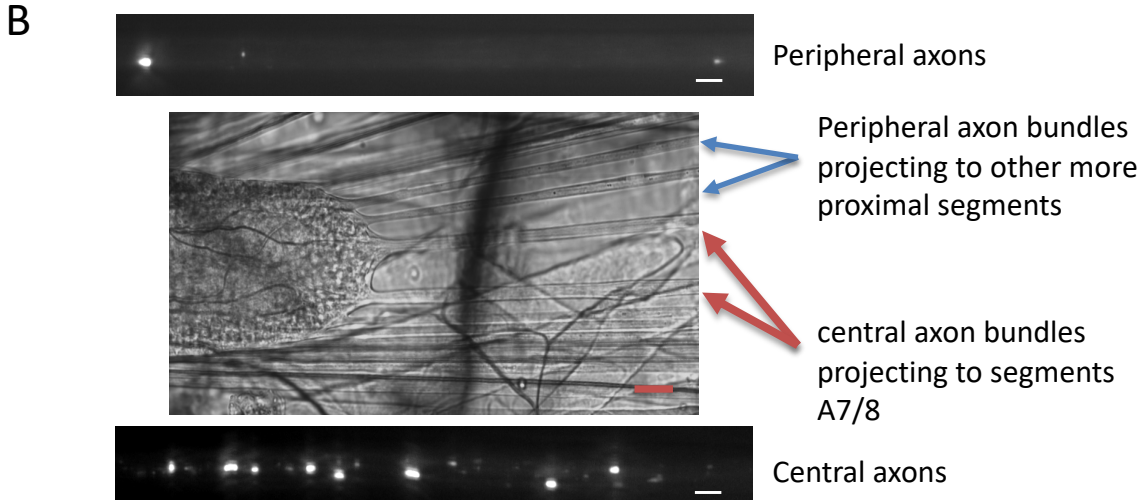
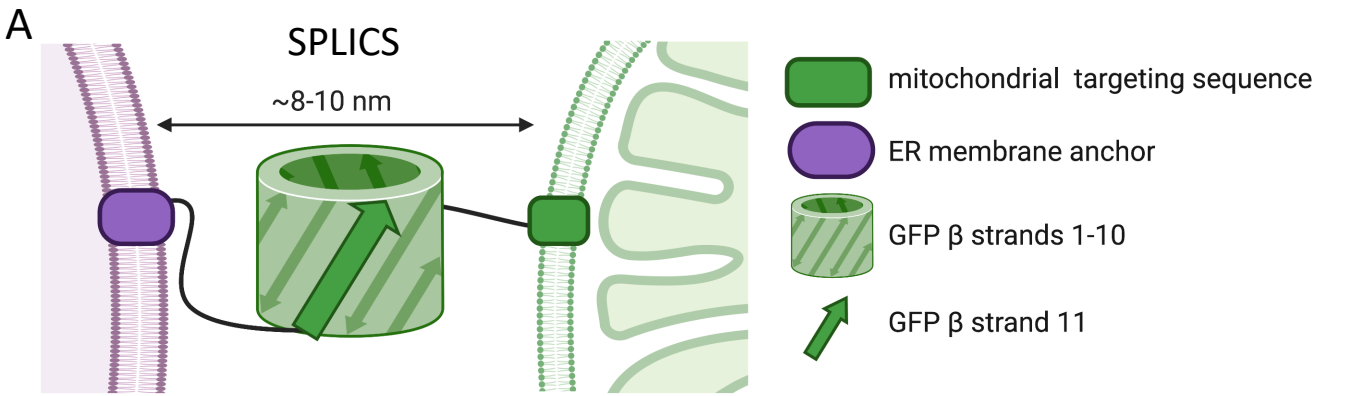


Figure S3. Phenotypic characterization of altered tethering in motor neurons.

